INFECTION AND IMMUNITY, Apr. 2004, p. 2429–2433 Vol. 72, No. 4
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.4.2429–2433.2004

Different Responses of Macrophages to Smooth and Rough *Brucella* spp.: Relationship to Virulence

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Received 3 November 2003/Returned for modification 14 November 2003/Accepted 11 December 2003

By comparing smooth wild-type *Brucella* strains to their rough mutants, we show that the lipopolysaccharide (LPS) O side chain of pathogenic *Brucella* has a dramatic impact on macrophage activation. It favors the development of virulent *Brucella* by preventing the synthesis of immune mediators, important for host defense. We conclude that this O chain property is firmly linked to *Brucella* virulence.

The genus *Brucella* is a gram-negative, facultative, intracellular pathogen that produces diseases in different mammals, including humans. As in other gram-bacteria, the lipopolysaccharide (LPS) of *Brucella* is an important component of the outer membrane (3, 7), but its precise role in the biology of the pathogen is still an unsolved issue. In smooth pathogenic *Brucella* spp. (*B. abortus*, *B. suis*, and *B. melitensis*), lipopolysaccharide (LPS) has been implicated in bacterial virulence. This proposal is based on the observation that rough mutants derived from these strains are greatly attenuated in animals or isolated macrophages compared to parental *Brucella* (1, 10, 13, 18, 33, 36, 37, 40). The rough mutants thus appear to act as a putative live vaccine. Their attenuation is mainly explained by the properties of the LPS O side chain, a long linear homopolymer of α1,2-linked perosamine (5). The O chain appears to be a key molecule for invasion and development (35) and protection from apoptosis (14). It also protects the bacteria from cellular cationic peptides (16, 32), oxygen metabolites (39), and complement-mediated lysis (13, 30). Recently, it was observed that the O chain also impairs cytokine production in infected human macrophages, and it was postulated that this could be a way for the pathogen to control host defense (37). We have analyzed this possibility in a murine model of infection commonly used to compare the levels of virulence of *Brucella* strains.

B. melitensis B3B2 (18) and R5 (Table 1) and *B. suis* manb (15) are three rough mutants of wild-type *B. melitensis* 16M and *B. suis* 1330, respectively: these mutants are attenuated in BALB/c mice compared to parental *Brucella* (9) (Table 1). Their ability to infect murine macrophage-like cells was assessed by using J774A.1 cells cultured in 24-well plates (10^6 cells per well). These cells were incubated at 37°C for 30 min with a bacterial suspension (multiplicity of infection [MOI] = 40) (21, 40). After three washes, the infected macrophages were reincubated in 1 ml of RPMI–10% fetal calf serum (FCS) supplemented with 30 μg of gentamicin/ml for at least 40 min to kill extracellular bacteria. At several intervals postinfection (p.i.), cells were washed and lysed in 0.2% Triton X-100. The number of viable intracellular bacteria (CFU per well) was determined by plating serial 10-fold dilutions onto Trypticase soy agar (TSA) plates. Figure 1 indicates that rough *B. melitensis* strains R5 and B3B2 were respectively phagocytosed 500- and 100-fold more than smooth *B. melitensis* strain 16M ($P < 0.005$ for each mutant versus *B. melitensis*) and that *B. suis* manb was internalized 50-fold more than *B. suis* 1330 ($P < 0.003$). As reported previously (18, 21, 24), after a short period of decrease, the number of intracellular *B. suis* and *B. melitensis* cells significantly increased. At 48 h p.i., there were 100- to 1,000-fold more intracellular smooth bacteria than were found at the onset of infection. In contrast, intracellular rough mutants were eliminated, and depending on the mutant analyzed, there were 10^2- to 10^3-fold-fewer intracellular bacteria at 48 h p.i. than after phagocytosis. All of the rough mutants were eliminated, albeit with different kinetics, which can be explained by the genetic background of the mutants. The elevated invasion of the rough mutants was possibly due to the exposure of ligands that are normally hidden by the O chain and the consequent increased capacity of rough *Brucella* to adhere to macrophages (11, 37). Entry of smooth and rough *Brucella* strains into the cells through different pathways (35) could also involve receptors with a distinct ability to regulate the levels of phagocytosis. Because rough *Brucella* strains are efficiently internalized (10, 11, 17, 35), the bacteria could alter the plasma membrane, causing cell damage. Cell toxicity could also have resulted from induction of cell apoptosis, because rough *Brucella* strains do not protect macrophages from exogenous apoptotic signals (14), unlike smooth *Brucella* strains (23). However, under our experimental conditions (MOI of 40, presence of serum, no exogenous apoptotic signals, 48 h of culture), the lactate dehydrogenase activities of supernatants (measured as desrcribed in reference 22) were similar in cells infected by rough or smooth *Brucella* strains (data not shown). This indicates that elimination of the rough mutant did not result from cell toxicity and release of bacteria in the gentamicin-supplemented medium. Therefore, as postulated (37), the observed fate of rough *Brucella* strains could have resulted from a cellular response triggered by the bacteria. The macrophage response to different *Brucella* was assessed by measuring the expression of inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-

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TABLE 1. *Brucella* strains used in the study

Strain	Genotypic and/or phenotypic description[a]	No. of CFU/ spleen at 3 wk p.i.[b]
B. melitensis		
16M	Spontaneous smooth nalidixic acid-resistant mutant of *B. melitensis* 16M, (41)	$>10^4$
R5	Natural rough mutant of *B. melitensis* 16M (SIA-DGA collection, Zaragoza, Spain)	<10
B3B2	Rough mini-Tn*5* insertion mutant of *B. melitensis* 16M with the perosamine synthase gene deleted, kanamycin resistant (18)	<10
B. suis		
1330	Smooth wild-type *B. suis* (ATCC 23444)	$>5 \times 10^5$
manb	Rough mini-Tn*5* insertion mutant of *B. suis* 1330 with the phosphomannose gene deleted, kanamycin resistant (15)	<10
GFP	Smooth ampicillin-resistant mutant of *B. suis* 1330 that constitutively expresses the GFP gene (32)	$>5 \times 10^5$

[a] The smooth and rough phenotypes of the different *Brucella* strains were assessed by crystal violet staining and verified by immunoblotting techniques involving monoclonal antibodies that recognize the smooth or rough LPS of *Brucella* (8).

[b] BALB/c mice were injected intraperitoneally with 5×10^4 CFU of *B. suis* 1330 or manb or of *B. melitensis* 16M, B3B2, or R5. Six mice per group were killed by CO_2 asphyxiation 3 weeks post-inoculation. Spleens were aseptically removed and homogenized with 10 ml of phosphate-buffered saline to determine *Brucella* counts (CFU per spleen) as indicated in references 28 and 29.

10, IL-12, MIP-2, and KC (CXC chemokine, murine homologue of GRO-alpha) mRNAs in cells infected for 5 h. Expression of mRNAs was determined by reverse transcription-PCR (RT-PCR) under the conditions described in our previous studies (20, 21). The primers, mRNA and cDNA preparations, and procedures used for normalization of amplicon intensities have been described elsewhere in detail (20, 21, 24). The inflammatory cytokine and iNOS transcripts that are not expressed in quiescent cells were strongly induced when the invasive agent was one of the rough bacteria, but not when *B. suis* or *B. melitensis* 16M was used. This indicated that rough *Brucella* strains triggered an activation process that did not occur in smooth *Brucella* strain-infected cells. The transcripts were expressed at levels comparable to those occurring in quiescent cells stimulated with 100 ng of *Escherichia coli* LPS per ml. TNF-α and NO are two products of macrophage response directly involved in killing of intracellular *Brucella* (2, 9, 21, 42, 43); their production was measured in infected cell supernatants as described in reference 21. A significant accumulation of NO_2^- (the end product of NO) and TNF-α was observed in supernatants of all rough *Brucella* strain-infected cells. The accumulation of NO was time dependent for at least 48 h p.i. and was similar to that induced by the combination of *E coli* LPS with gamma interferon (IFN-γ) (Table 2) (21). The TNF-α concentration, optimal at 24 h, varied from 10 to 28 ng/ml, depending on the rough mutant. In accordance with previous reports (12, 21, 41), macrophages invaded by smooth *Brucella* strains did not express iNOS nor release any NO (Table 2). They produced relatively weak amounts of TNF-α, the cytokine concentration in supernatants remaining around 0.5 ng/ml (Table 2), which in each case was significantly lower than that induced by each of the rough bacterial strains assessed. Several studies have clearly demonstrated an inverse correlation between TNF-α release and the invasive capacity of *Brucella* (reviewed in reference 2). Moreover, NO which is deleterious to *Brucella* (28), was produced in rough *Brucella* strain-infected cells but not in smooth *Brucella* strain-infected cells. Besides TNF-α and NO, IL-1 is an important mediator of *Brucella* development (27), and the phagolysosomal destruction of microorganisms commonly corresponds to high levels of cytokine production (34). KC and MIP-2 have antibacterial properties in vivo by attracting neutrophils to the site of infection (24, 34), and IL-12 is a necessary factor for the establish-

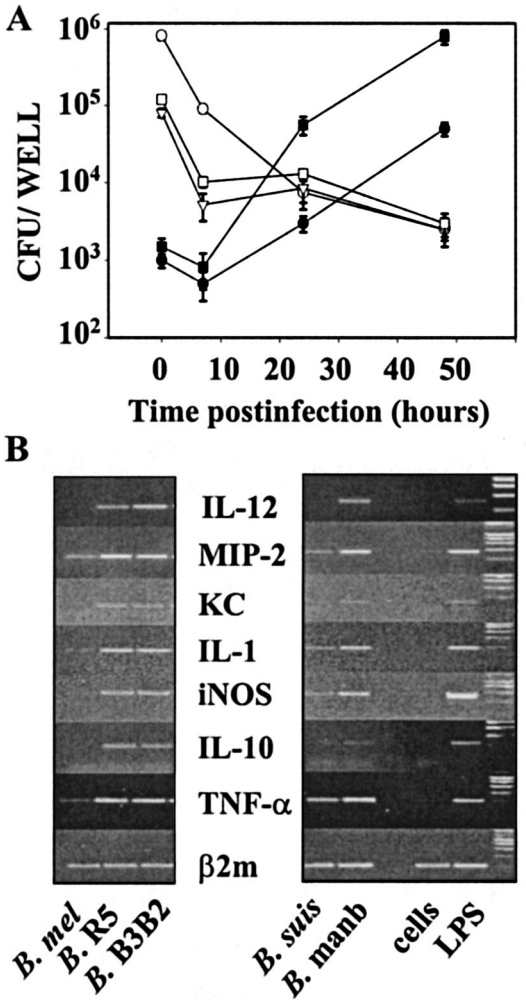

FIG. 1. (A) Infection of J774.A1 cells by different smooth and rough strains of *Brucella*. J774.A1 cells were infected (MOI = 40) with *B. suis* (■), *B. suis* manb (□), *B. melitensis* 16M (●), *B. melitensis* B3B2 (▽), or *B. melitensis* R5 (○), and the intracellular fate of the bacteria was evaluated. The data presented are means ± standard deviations of triplicate plate counts and are representative of three different experiments. (B) Cytokine and iNOS mRNA expression in *Brucella*-infected cells. The gene expression of different cytokines or iNOS was analyzed by RT-PCR performed on mRNAs of J774.A1 cells infected for 5 h and compared to gene expression in control cells (cells) or in cells induced with 100 ng/*E. coli* LPS/ml. The housekeeping gene coding for β2-microglobulin was used as a standard (31). Cells were infected with *B. melitensis* 16M, R5, or B3B2 or *B. suis* 1330 or manb. Data are representative of three different experiments. The mRNAs and cDNA preparations, primers, and method used to compare amplicon intensities have been described elsewhere in detail (28, 31).

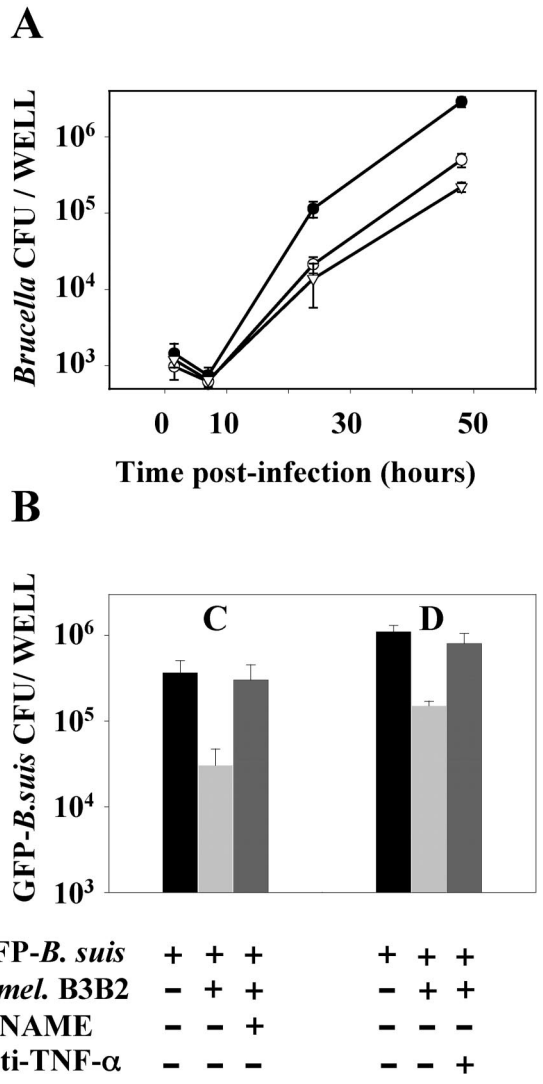

FIG. 2. (A) Intracellular development of *B. suis* GFP (CFU per well) in macrophages infected with *B. suis* GFP or coinfected with *B. suis* GFP and a rough mutant of *Brucella*. J774.A1 cells (10^6/well) were infected with *B. suis* GFP (MOI = 40) (●) or coinfected with *B. suis* GFP and *B. suis* manb (○) or *B. suis* GFP and *B. melitensis* B3B2 (▽) (MOI = 40 for each bacteria). Infections were performed in triplicate. At different periods of time, the cells were lysed and the number of intracellular *B. suis* GFP cells was determined by plating the cell lysates on agar plates supplemented with ampicillin and expressed in CFU per well ± standard deviation. At 48 h p.i., in four different experiments, the number of *B. suis* GFP cells in macrophages infected with only *B. suis* GFP was significantly higher than the number of *B. suis* GFP cells in macrophages coinfected with *B. suis* GFP and *B. suis* manb ($P < 0.05$) or with *B. suis* GFP and *B. melitensis* B3B2 ($P < 0.01$). (B) Effect of NO and TNF-α on the development of *B. suis* in J774.A1 cells coinfected with *B. suis* and *B. melitensis* B3B2. J774.A1 cells (10^6/well) were infected with *B. suis* GFP (MOI = 40) or coinfected with *B. suis* GFP and *B. melitensis* B3B2 (MOI = 40, for each bacteria). After the phagocytosis step (i.e., at the same time as gentamicin), L-NAME (3 mM) or the blocking anti-TNF-αR antibody (5 μg/ml) was added (or not) to the infected cultures. Infections were performed in triplicate. Forty-eight hours later, the intracellular number of *B. suis* GFP cells was determined for each condition and expressed as CFU per well ± standard deviation. Experiments C and D were performed separately and repeated four times. The numbers of *B. suis* GFP cells phagocytosed in 30 min were as follows: 1,200 ± 400 (inoculum of *B. suis* GFP) and 1,500 ± 200 (inoculum of *B. suis* GFP and *B. melitensis* B3B2) for experiment C and 2,200 ± 500 (inoculum of *B. suis* GFP) and 1,900 ± 300 (inoculum of *B. suis* GFP and *B. melitensis* B3B2) for experiment D. In four separate experiments, at 48 h p.i., the numbers of intracellular *B. suis* GFP cells were (i) significantly lower in coinfections than in macrophages infected with only *B. suis* GFP ($P < 0.001$ and $P < 0.01$ for experiments C and D, respectively), (ii) significantly higher in coinfections performed in the presence of L-NAME than in its absence ($P < 0.05$), and (iii) significantly higher in coinfections performed in the presence of anti-TNF-αR than in its absence ($P < 0.01$).

TABLE 2. NO_2^- and TNF-α production in J774.A1 cells infected with different strains of *Brucella* or coinfected with *B. suis* GFP and a *Brucella* rough mutant

Infection[a]	Production of[b]:	
	NO_2^- at 48 h (μM)	TNF-α at 24 h (ng/ml)
B. suis 1330	1.9 ± 0.5*	0.7 ± 0.5*
B. suis manb	27.7 ± 1.7†‡	28.7 ± 1.5†‡
B. melitensis 16M	3.0 ± 1.1*	0.5 ± 0.3*
B. melitensis R5	22.0 ± 3‡§	10.0 ± 2‡§
B. melitensis B3B2	16.3 ± 3.7‡§	30.7 ± 1.4‡§
B. suis GFP	2.3 ± 0.5*	0.5 ± 0.2*
B. suis GFP + manb	24.8 ± 0.9‖	24.9 ± 5.3‖
B. suis GFP + *B. melitensis* B3B2	18.2 ± 3.7‖	27.8 ± 2.5‖
E. coli LPS + IFN-γ	43.2 ± 3.1‡	37.5 ± 3.1‡
None	0.2 ± 0.2	0.7 ± 0.4

[a] J774.A1 cells (10^6/well) were infected (or not) with different smooth or rough *Brucella* strains (MOI = 40) or cultured with 100 ng of *E. coli* LPS plus 2 U of mouse recombinant IFN-γ per ml. In parallel experiments, cells were infected with *B. suis* GFP (MOI = 40) in the absence or presence of *B. suis* manb (or *B. melitensis* B3B2) (MOI = 40). At the indicated periods of time, cell supernatants were harvested and their NO_2^- or TNF-α contents were determined (21). The data presented are means ± standard deviations of three different experiments. Comparisons between different assays were performed by using unpaired Student's *t* tests.

[b] *, not significant compared to nontreated cells; †, $P < 0.001$ compared to *B. suis* 1330; ‡, $P < 0.001$ compared to nontreated cells; §, $P < 0.001$ compared to *B. melitensis* 16M; ‖, $P < 0.001$ compared with values obtained in infections with *B. suis* GFP alone.

ment of the Th1 response, which in vivo determines the elimination of *Brucella* (44). If IL-10 is detrimental to the cells, it is also an anti-inflammatory molecule that controls the damaging effect of proinflammatory cytokines.

Therefore, in rough *Brucella* strain infection, the high production of TNF-α and NO concentration and possibly that of other effectors may mount a potent inflammatory response that imbalances the macrophage defense mechanisms to an extent favorable to the host cells. Conversely, smooth *Brucella* strains could be virulent in part because they induce little or no macrophage response, the LPS O chain limiting this response. To confirm this possibility, J774.A1 macrophages were coinfected with smooth and rough *Brucella* strains, and the proliferation of the smooth bacteria was measured. J774.A1 cells (10^6/ml/well) were simultaneously incubated at 37°C with *B. suis* GFP (an ampicillin-resistant mutant of *B. suis* 1330 that expresses the green fluorescent protein [GFP] gene) instead of *B. suis* (MOI = 40) (31) and a rough strain of *Brucella* (*B. suis* manb or *B. melitensis* B3B2) (MOI = 40) for 30 min. The protocol commonly used for infection (Fig. 1) (washing, cultures grown in RPMI-FCS with gentamicin, cell lysis with 0.2% Triton X-100 at different times p.i., and determination of CFU) was then applied. J774. A1 cells were also infected with

B. suis GFP alone (MOI = 40) or with one of the rough mutants (MOI = 40). To measure only the development of *B. suis* GFP, and not that of the rough strain, cell lysates were applied to TSA plates containing 50 μg of ampicillin per ml. *B. suis* GFP carries a stable resistance gene to this antibiotic (39), while rough *Brucella* strains did not proliferate on these plates. Figure 2A shows that the phagocytosis of *B. suis* GFP was not affected by the presence of *B. suis* manb or *B. melitensis* B3B2. In parallel, the phagocytosis of the rough strain was not modified in presence of *B. suis* (data not shown). These observations agreed with the different pathways of entry of smooth and rough *Brucella* strains (35). At 24 and 48 h p.i., the number of intracellular *B. suis* GFP organisms was significantly lower within macrophages infected with both strains than within macrophages infected with *B. suis* GFP alone (Fig. 2A). In coinfected cells, NO_2^- and TNF-α accumulated in supernatants of cells simultaneously infected with *B. suis* GFP and *B. suis* manb (or *B. melitensis* B3B2), but not (or poorly for TNF-α) in *B. suis* GFP-infected cells. These accumulations were similar to those occurring in supernatants of cells solely infected by rough mutants ($P > 0.2$) (Table 2). To study whether NO and TNF-α affect the fate of *B. suis* GFP, the iNOS inhibitor L-NAME (*N*-ω-nitro-L-arginine methly ester) (21) or a blocking anti-TNF-α receptor (TNF-αR) antibody was assessed in coinfection experiments. L-NAME does not affect the infection of J774.A1 cells with only *B. suis* (21). On the contrary, it reversed the inhibition of the intracellular development of *B. suis* GFP induced by the rough mutant and favored the growth of the pathogen (Fig. 2C). The anti-TNF-αR antibody also exerted a similar effect (Fig. 2D). The concomitant measurement of NO_2^- in cell supernatants confirmed the inhibitory effect of L-NAME on NO production. At 48 h p.i., NO_2^- concentrations in the presence and absence of L-NAME were 4.25 ± 1 and 37 ± 5 μM, respectively, in coinfections involving *B. suis* GFP and *B. melitensis* B3B2. As expected (6, 21), in control cells at 48 h p.i., L-NAME (20-fold) and the anti-TNF-αR antibody (5-fold) positively affected the development of *B. melitensis* B3B2 (data not shown). Altogether, the experiments finally indicated that TNF-α and NO resulting from a response triggered by rough bacteria inhibited the development of *B. suis* GFP. In the presence of L-NAME or anti-TNF-αR antibody, the number of *B. suis* GFP cells in coinfected cells did not differ from that in cells infected with *B. suis* GFP alone. Therefore, when the NO or TNF-α effect is neutralized, the intracellular development of *B. suis* GFP was not affected by the presence of rough bacteria within the cells. Therefore, the inhibition of *B. suis* GFP development was not due to (i) too large an ingestion of rough bacteria by macrophages that could have damaged the cells, nor (ii) a rerouting of the bacteria towards phagolysosomes, because the level of uptake of *B. suis* was not significantly affected by the uptake of the rough strain and vice versa, a result in accordance with the different routes of entry of smooth and rough *Brucella* strains (35). Finally, the impairment of the development of *B. suis* GFP reversed by L-NAME or anti-TNF-αR resulted from a direct response of the macrophage to rough *Brucella* strains. Therefore, smooth *Brucella* strains might achieve long-term persistence, because they do not initiate the production of NO and TNF-α, two factors that induce the clearance of rough *Brucella.* This may also be true for other inflammatory molecules at different stages of infection.

B. abortus binds to several different receptors, including LPS receptors (4). However, the LPS from both rough and smooth *Brucella* strains is a weak activator of macrophages (19, 26). Therefore, the elevated expression of cytokine and iNOS transcripts in rough *Brucella*-infected macrophages compared to macrophages stimulated by *E. coli* LPS could mean that the bacterial LPS is not the only molecule involved in macrophage stimulation. Such a possibility is in agreement with observations showing that TLR-2, but not TLR-4 which is involved in LPS response, mediates macrophage activation by heat killed *Brucella* (25). This should explain how rough *Brucella* strains are able to trigger the complete cell signaling pathways leading to NO production, while LPS-induced production of NO requires an additional signal given by exogenous IFN-γ (21). In accordance with previous reports comparing *B. abortus* 2308 and *B. abortus* RB51 (38), *B. melitensis* and its *whoA* mutant (13), or *B. suis* and its rough mutants (35), observations from our group indicate that many more receptors are engaged in macrophage response to rough *Brucella* infection than in response to smooth *Brucella* infection (35, 37). This could explain the potent capacity of rough mutants to stimulate macrophages. The different pathways of phagocytosis of rough and smooth bacteria suggest that these bacteria engage different sets of receptors (35). Therefore, besides the number of receptors engaged, their specificity is certainly of importance. In any case, the slight response associated with smooth *Brucella* infection indicates a mechanism by which the LPS O chain modulates the host immune response to the pathogen's advantage. Besides its properties mentioned above (13, 14, 16, 30, 32, 35, 39), this O chain characteristic must be taken into account to explain the virulence of pathogenic *Brucella*. The cell signals by which smooth *Brucella* avoids macrophage activation at the molecular level are now under investigation.

These studies were supported by grants from INSERM and from the European Community (QLK2-1999-0014). We are grateful for support from the Region Languedoc-Roussillon and Aragón (CTP FDG/CS68 for INSERM and CTPMO1/2002 for Aragón) and the Ministerio de Educación Cultura y Deporte: Programa de Estancias de Investigadores en el Extranjero PR2001-0053 and INSERM (Poste Vert) for exchange grants to María P. Jiménez de Bagüés.

We thank A. Cloeckaert (INRA, France) for providing the LPS monoclonal antibodies and F. Godfroid (Université de Namur, Namur, Belgium) for the *B. melitensis* B3B2 mutant.

We thank Sue Hagius and E. Caron for correcting and improving our English.

REFERENCES

1. **Allen, C. A., L. G. Adams, and T. A. Ficht.** 1998. Transposon-derived *Brucella abortus* rough mutants are attenuated and exhibit reduced intracellular survival. Infect. Immun. **66:**1008–1016.
2. **Baldwin, C. L., and M. Parent.** 2002. Fundamentals of host immune response against *Brucella* abortus: what the mouse model has revealed about control of infection. Vet. Microbiol. **90:**367–382.
3. **Bundle, D. R., J. W. Cherwonogrodzky, and M. B. Perry.** 1987. The structure of the lipopolysaccharide O-chain (M antigen) and polysaccharide B produced by *Brucella melitensis* 16M. FEBS Lett. **216:**261–264.
4. **Campbell, G. A., L. G. Adams, and B. A. Sowa.** 1994. Mechanisms of binding of *Brucella abortus* to mononuclear phagocytes from cows naturally resistant or susceptible to brucellosis. Vet. Immunol. Immunopathol. **41:**295–306.
5. **Caroff, M., D. R. Bundle, and M. B. Perry.** 1984. Structure of the O-chain of the phenol-phase soluble cellular lipopolysaccharide of *Yersinia enterocolitica* serotype O:9. Eur. J. Biochem. **139:**195–200.
6. **Caron, E., T. Peyrard, S. Köhler, S. Cabane, J.-P. Liautard, and J. Dornand.**

1994. Live *Brucella* spp. fail to induce tumor necrosis factor alpha excretion in U937-derived phagocytes. Infect. Immun. **62:**5267–5274.
7. **Cherwonogrodzky, J. W., M. B. Perry, and D. R. Bundle.** 1987. Identification of the A and M antigens of *Brucella* as the O-polysaccharides of smooth lipopolysaccharides. Can. J. Microbiol. **33:**979–981.
8. **Cloeckaert, A., P. de Wergifosse, G. Dubray, and J. N. Limet.** 1990. Identification of seven surface-exposed *Brucella* outer membrane proteins by use of monoclonal antibodies: immunogold labeling for electron microscopy and enzyme-linked immunosorbent assay. Infect. Immun. **58:**3980–3987.
9. **Corbeil, L. B., K. Blau, T. J. Inzana, K. H. Nielsen, R. H. Jacobson, R. R. Corbeil, and A. J. Winter.** 1988. Killing of *Brucella abortus* by bovine serum. Infect. Immun. **56:**3251–3261.
10. **Detilleux, P. G., B. L. Deyoe, and N. F. Cheville.** 1990. Entry and intracellular localization of *Brucella* spp. in Vero cells: fluorescence and electron microscopy. Vet. Pathol. **27:**317–328.
11. **Detilleux, P. G., B. L. Deyoe, and N. F. Cheville.** 1990. Penetration and intracellular growth of *Brucella abortus* in nonphagocytic cells in vitro. Infect. Immun. **58:**2320–2328.
12. **Eze, M. O., L. Yuan, R. M. Crawford, C. M. Paranavitana, T. L. Hadfield, A. K. Bhattacharjee, R. L. Warren, and D. L. Hoover.** 2000. Effects of opsonization and gamma interferon on growth of *Brucella melitensis* 16M in mouse peritoneal macrophages in vitro. Infect. Immun. **68:**257–263.
13. **Fernandez-Prada, C. M., M. Nikolich, R. Vemulapalli, N. Sriranganathan, S. M. Boyle, G. G. Schurig, T. L. Hadfield, and D. L. Hoover.** 2001. Deletion of *wboA* enhances activation of the lectin pathway of complement in *Brucella abortus* and *Brucella melitensis.* Infect. Immun. **69:**4407–4416.
14. **Fernandez-Prada, C. M., E. B. Zelazowska, M. Nikolich, T. L. Hadfield, R. M. Roop II, G. L. Robertson, and D. L. Hoover.** 2003. Interactions between *Brucella melitensis* and human phagocytes: bacterial surface O-polysaccharide inhibits phagocytosis, bacterial killing, and subsequent host cell apoptosis. Infect. Immun. **71:**2110–2119.
15. **Foulongne, V., G. Bourg, C. Cazevieille, S. Michaux-Charachon, and D. O'Callaghan.** 2000. Identification of *Brucella suis* genes affecting intracellular survival in an in vitro human macrophage infection model by signature-tagged transposon mutagenesis. Infect. Immun. **68:**1297–1303.
16. **Freer, E., E. Moreno, I. Moriyón, J. Pizarro-Cerdá, A. Weintraub, and J.-P. Gorvel.** 1996. *Brucella-Salmonella* lipopolysaccharide chimeras are less permeable to hydrophobic probes and more sensitive to cationic peptides and EDTA than are their native *Brucella* sp. counterparts. J. Bacteriol. **178:**5867–5876.
17. **Freer, E., J. Pizarro-Cerdá, A. Weintraub, J.-A. Bengoechea, I. Moriyón, K. Hultenby, J.-P. Gorvel, and E. Moreno.** 1999. The outer membrane of *Brucella ovis* shows increased permeability to hydrophobic probes and is more susceptible to cationic peptides than are the outer membranes of mutant rough *Brucella abortus* strains. Infect. Immun. **67:**6181–6186.
18. **Godfroid, F., B. Taminiau, I. Danese, P. Denoel, A. Tibor, V. Weynants, A. Cloeckaert, J. Godfroid, and J.-J. Letesson.** 1998. Identification of the perosamine synthetase gene of *Brucella melitensis* 16M and involvement of lipopolysaccharide O side chain in *Brucella* survival in mice and in macrophages. Infect. Immun. **66:**5485–5493.
19. **Goldstein, J., T. Hoffman, C. Frasch, E. F. Lizzio, P. Beining, D. Hochstein, Y. L. Lee, R. D. Angus, and B. Golding.** 1992. Lipopolysaccharide (LPS) from *Brucella abortus* is less toxic than that from *Escherichia coli*, suggesting the possible use of *B. abortus* or LPS from *B. abortus* as a carrier in vaccines. Infect. Immun. **60:**1385–1389.
20. **Gross, A., N. Dugas, S. Spiesser, I. Vouldoukis, C. Damais, J. P. Kolb, B. Dugas, and J. Dornand.** 1998. Nitric oxide production in human macrophagic cells phagocytizing opsonized zymosan: direct characterization by measurement of the luminol dependent chemiluminescence. Free Radic. Res. **28:**179–191.
21. **Gross, A., S. Spiesser, A. Terraza, B. Rouot, E. Caron, and J. Dornand.** 1998. Expression and bactericidal activity of nitric oxide synthase in *Brucella suis*-infected murine macrophages. Infect. Immun. **66:**1309–1316.
22. **Gross, A., A. Terraza, J. Marchant, S. Bouaboula, S. Ouahrani, J. P. Liautard, P. Casellas, and J. Dornand.** 2000. A beneficial aspect of a CB1 cannabinoid receptor antagonist: SR141716A is a potent inhibitor of macrophage infection by the intracellular pathogen *Brucella suis.* J. Leukoc. Biol. **67:**335–344.
23. **Gross, A., A. Terraza, S. Ouahrani-Bettache, J.-P. Liautard, and J. Dornand.** 2000. In vitro *Brucella suis* infection prevents the programmed cell death of human monocytic cells. Infect. Immun. **68:**342–351.
24. **Guilloteau, L. A., J. Dornand, A. Gross, M. Olivier, F. Cortade, Y. Le Vern, and D. Kerboeuf.** 2003. Nramp1 is not a major determinant in the control of *Brucella melitensis* infection in mice. Infect. Immun. **71:**621–628.
25. **Huang, L.-Y., J. Aliberti, C. A. Leifer, D. M. Segal, A. Sher, D. T. Golenbock, and B. Golding.** 2003. Heat-killed *Brucella abortus* induces TNF and IL-12p40 by distinct MyD88-dependent pathways: TNF, unlike IL-12p40 secretion, is toll-like receptor 2 dependent. J. Immunol. **171:**1441–1446.
26. **Jarvis, B. W., T. H. Harris, N. Qureshi, and G. A. Splitter.** 2002. Rough lipopolysaccharide from *Brucella abortus* and *Escherichia coli* differentially activates the same mitogen-activated protein kinase signaling pathways for tumor necrosis factor alpha in RAW 264.7 macrophage-like cells. Infect. Immun. **70:**7165–7168.
27. **Jiang, X., and C. L. Baldwin.** 1993. Effects of cytokines on intracellular growth of *Brucella abortus.* Infect. Immun. **61:**124–134.
28. **Jimenez de Bagües, M. P., C. M. Marin, M. Barberan, and J. M. Blasco.** 1993. Evaluation of vaccines and of antigen therapy in mouse model for *Brucella ovis.* Vaccine **11:**61–66.
29. **Jiménez de Bagüés, M. P., P. H. Elzer, J. M. Blasco, C. M. Marin, C. Gamazo, and A. J. Winter.** 1994. Protective immunity to *Brucella ovis* in BALB/c mice following recovery from primary infection or immunization with subcellular vaccines. Infect. Immun. **62:**632–638.
30. **Joiner, K. A.** 1985. Studies on the mechanism of bacterial resistance to complement-mediated killing and on the mechanism of action of bactericidal antibody. Curr. Top. Microbiol. Immunol. **121:**99–133.
31. **Köhler, S., S. Ouahrani-Bettache, M. Layssac, J. Teyssier, and J.-P. Liautard.** 1999. Constitutive and inducible expression of green fluorescent protein in *Brucella suis.* Infect. Immun. **67:**6695–6697.
32. **Martinez de Tejada, G., J. Pizarro-Cerda, E. Moreno, and I. Moriyón.** 1995. The outer membranes of *Brucella* spp. are resistant to bactericidal cationic peptides. Infect. Immun. **63:**3054–3061.
33. **McQuiston, J. R., R. Vemulapalli, T. J. Inzana, G. G. Schurig, N. Sriranganathan, D. Fritzinger, T. L. Hadfield, R. A. Warren, N. Snellings, D. Hoover, S. M. Halling, and S. M. Boyle.** 1999. Genetic characterization of a Tn*5*-disrupted glycosyltransferase gene homolog in *Brucella abortus* and its effect on lipopolysaccharide composition and virulence. Infect. Immun. **67:**3830–3835.
34. **Murdoch, C., and A. Finn.** 2000. Chemokine receptors and their role in inflammation and infectious diseases. Blood **95:**3032–3043.
35. **Porte, F., A. Naroeni, S. Ouahrani-Bettache, and J.-P. Liautard.** 2003. Role of the *Brucella suis* lipopolysaccharide O antigen in phagosomal genesis and in inhibition of phagosome-lysosome fusion in murine macrophages. Infect. Immun. **71:**1481–1490.
36. **Riley, L. K., and D. C. Robertson.** 1984. Brucellacidal activity of human and bovine polymorphonuclear leukocyte granule extracts against smooth and rough strains of *Brucella abortus.* Infect. Immun. **46:**231–236.
37. **Rittig, G., A. Kaufmann, A. Robins, B. Shaw, H. Sprenger, D. Gemsa, V. Foulongne, B. Rouot, and J. Dornand.** 2003. Smooth and rough lipopolysaccharide phenotypes of *Brucella* induce different intracellular trafficking and cytokine/chemokine release in human monocytes. J. Leukoc. Biol. **74:**1045–1055.
38. **Sola-Landa, A., J. Pizarro-Cerda, M. J. Grillo, E. Moreno, I. Moriyon, J. M. Blasco, J. P. Gorvel, and I. Lopez-Goni.** 1998. A two-component regulatory system playing a critical role in plant pathogens and endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence. Mol. Microbiol. **29:**125–138.
39. **Stinavage, P., L. E. Martin, and J. K. Spitznagel.** 1989. O antigen and lipid A phosphoryl groups in resistance of *Salmonella typhimurium* LT-2 to non-oxidative killing in human polymorphonuclear neutrophils. Infect. Immun. **57:**3894–3900.
40. **Ugalde, J. E., C. Czibener, M. F. Feldman, and R. A. Ugalde.** 2000. Identification and characterization of the *Brucella abortus* phosphoglucomutase gene: role of lipopolysaccharide in virulence and intracellular multiplication. Infect. Immun. **68:**5716–5723.
41. **Verger, J. M., M. Grayon, E. Chaslus-Dancla, M. Meurisse, and J. P. Lafont.** 1993. Conjugative transfer and in vitro/in vivo stability of the broad-host-range IncP R751 plasmid in *Brucella* spp. Plasmid **29:**142–146.
42. **Wang, M., N. Qureshi, N. Soeurt, and G. Splitter.** 2001. High levels of nitric oxide production decrease early but increase late survival of *Brucella abortus* in macrophages. Microb. Pathog. **31:**221–230.
43. **Zhan, Y., and C. Cheers.** 1998. Control of IL-12 and IFN-γ production in response to live or dead bacteria by TNF and other factors. J. Immunol. **161:**1447–1453.
44. **Zhan, Y. F., E. R. Stanley, and C. Cheers.** 1991. Prophylaxis or treatment of experimental brucellosis with interleukin-1. Infect. Immun. **59:**1790–1794.

Editor: D. L. Burns